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(71) Applicant *(for all designated States except US)*: COMMONWEALTH SERUM LABORATORIES COMMISSION [AU/AU]; 45 Poplar Road, Parkville, VIC 3052 (AU).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : GEYSEN, Hendrik, Mario [AU/AU]; 10 Janden Close, Knoxfield, VIC 3180 (AU).

(74) Agents: SLATTERY, John, Michael et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

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(54) Title: ANTIGENICALLY ACTIVE AMINO ACID SEQUENCES

(57) Abstract

A synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, characterised in that at least a portion of said peptide is selected from the group consisting of five-, six- or seven-long antigenically active amino acid sequences of said VP1 protein, and antigenically active modified sequences thereof.

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"ANTIGENICALLY ACTIVE AMINO ACID SEQUENCES"

This invention relates to the identification and chemical synthesis of peptides (or amino acid sequences) which constitute the immunogenic determinant(s) of an immunologically important coat protein, VP1, of foot-and-mouth disease virus, and to the use of these peptides for example, in the production of vaccines and diagnostic reagents.

In the search for more effective means of protection against infective disease in man and animals, major advances have been made in the last decade. It is now clear that immunization is possible using an isolated component of the whole causative agent, such as for example in the influenza virus sub-unit vaccines.

Current efforts are directed at reducing the scale of the immunizing component still further, first to the polypeptide (protein) carrying the necessary trigger for the immune system and secondly to the trigger itself. Recombinant DNA technology has provided the means, by translation from the determined nucleotide sequences, of obtaining reliable amino acid

sequences for biologically important proteins including such proteins which were not readily available from natural sources. However, methods for identifying the loci of a protein which constitute the trigger(s) or immunogenic determinant(s) are few and very time consuming and form the bottle neck to further rapid progress.

The determination of the immunogenic determinant(s) for biologically important proteins, particularly proteins derived from the causative agents of infective disease in man and animals, is regarded as being of particular importance since, once these determinants have been identified, they can be simply and economically synthesised so as to provide the desired peptide sequences for use in vaccines which will have a high degree of specificity, and which will avoid any undesired effects from unnecessary amino acid or peptide sequences which might still be present in, for example, sub-unit type vaccines.

The immunogenicity of a polypeptide can be defined as the immune response directed against a limited number of immunogenic determinants, which are confined to a few loci on the polypeptide molecule, (see Crumpton, M.J., in The Antigens (ed. Sela, M., Academic Press, New York, 1974); Benjamini, E. et al., Curr. Topics Microbiol. Immunol. 58, 85-135 (1972); and Atassi, M.Z., Immunochemistry 12, 423-438 (1975).) Antisera prepared against chemically

synthesized peptides corresponding to short linear stretches of the polypeptide sequence have been shown to react well with the whole polypeptide, (see Green, N. et al., Cell 28, 477-487 (1982); Bittle, J.L. et al., Nature 298, 30-33 (1982); Dreesman et al., Nature 295, 158-160 (1982); Prince, A.M., Ikram, H., Hopp, T.P., Proc. Nat. Acad.Sci. USA 79, 579-582 (1982); Lerner, R.A. et al., Proc.Nat.Acad.Sci.USA 78, 3403-3407 (1981); and Neurath, A.R., Kent, S.B.H., Strick, N., Proc.Nat.Acad.Sci. USA 79, 7871-7875 (1982).) However, interactions have been found to occur even when the site of interaction does not correlate with the immunogenic determinants of the native protein, (see Green, N., et al, Supra). Conversely, since antibodies produced against the native protein are by definition directed to the immunogenic determinants, it follows that a peptide interacting with these antibodies must contain at least a part of an immunogenic determinant.

From a study of the few proteins for which the immunogenic determinants have been accurately mapped, it is clear that a determinant can consist of a single sequence, (continuous), or of several sequences (discontinuous) brought together from linearly distant regions of the polypeptide chain by the folding of that chain as it exists in the native state, (see Atassi, M.Z., Immunochemistry 15, 909-936 (1978).). As in the case of lysozyme several of the elements consist of only one amino acid, the size of a contributing element can then vary between one and the

maximum number of amino acids consistent with the dimensions of the antibody combining site, and is likely to be of the order of five to six, (see Atassi, M.Z., supra). Any systematic mapping of all the detectable antigenic elements of a polypeptide by the chemical synthesis of overlapping segments and measurement of their subsequent reactivity with antisera prepared against the native protein has until now been severely limited by the scale of the synthetic and testing capability required, (see Atassi, M.Z., supra, and Kazim, A.L., Atassi, M.Z., Biochem.J. 191, 261-264 (1980)).

The precise localisation of immunogenic determinants within the amino acid sequence of a few proteins has been performed by one or more of the following approaches: (1) antigenicity measurements of the whole polypeptide or peptide fragments isolated therefrom, before and after chemical modification at specific residues; (2) locating the position, within the polypeptide amino acid sequence of substitutions, selected by growing the virus expressing the protein in the presence of monoclonal antibodies; and (3) synthesis and testing of peptides, homologous with the amino acid sequence, of regions suspected of immunogenic activity. This last method probably gives the best opportunities for a comprehensive approach; however, the synthesis and purification of numerous peptides requires a great deal of expertise and time. Smith, J.A., et al, Immunochemistry 14, 565-568 (1977), circumvented the decoupling and purification

steps by combining solid-phase peptide synthesis and solid-phase radio-immune assay using the same solid support, (see also Hurrell, J.G.R., Smith, J.A., Leach, S.J., Immunochemistry 15, 297-302 (1978)). With this procedure they were able to confirm the locations of the continuous immunogenic determinants of two proteins. However, even with this simplified approach, any systematic scan for antibody-binding activity of all possible hexapeptides from even a relatively small protein such as Virus Protein 1 (VP1) of foot-and-mouth disease virus (FMDV) which is known to have 213 amino acids, would take an unacceptably long time.

The FMD virus, which belongs to the aphthovirus genus of the family Picornaviridae, consists of an ordered aggregation of structurally independent sub-units surrounding a molecule of infectious single-stranded RNA. Under relatively mild conditions the whole particle readily dis-aggregates to give the naked RNA, 60 copies of the VP4 polypeptide, and 12 sub-units consisting of an ordered arrangement of five copies of each of the polypeptides, VP1, VP2 and VP3. Each of these structural sub-units can be further disrupted to yield the isolated component proteins. The VP1 protein of FMD virus has been shown to be an immunologically important coat protein of the virus.

Following the development of new techniques for the solid-phase synthesis of peptides, it has now

become possible to develop a method for the concurrent synthesis on solid supports of hundreds of peptides. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. A preferred method for solid-phase synthesis according to the present invention comprises the use of a polymeric material such as polyethylene or polypropylene as the solid-phase carrier, onto which is graft polymerised a vinyl monomer containing at least one functional group to produce polymeric chains on the carrier. The functional groups of these polymeric chains are then reacted to provide primary or secondary amino groups of the chains, and these amino groups are then sequentially reacted with amino acid residues in appropriate order so as to build up a desired synthetic peptide. The carrier is preferably in the form of a solid polymer rod having a diameter of about 4mm and a length of about 50mm. A number of such rods can be held in a suitable holder in a 12 x 8 grid whose dimensions correspond to those of the standard microtitre plate used for enzyme-linked immunosorbent assays (ELISA).

As a result of work now carried out using the above technique, peptides which constitute immunogenic determinant(s) of the VP1 coat proteins of a number of important serotypes of foot-and-mouth disease virus have been identified and chemically synthesized.

Note: Throughout this specification amino acid residues will be denoted by the three-letter abbreviation or single-letter code as follows:-

Amino Acid	Three-letter abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

In broad terms, the present invention provides a synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, characterised in that at least a portion of said peptide is selected from the group consisting of five-, six- or seven-long antigenically active amino acid sequences of said VP1 protein, and antigenically active modified sequences (as hereinafter defined) thereof.

According to a first aspect of the present invention, there is provided a synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, Type O_1, characterised in that at least a portion of said peptide is selected from amino acid sequences of the group consisting of:

(i) G - D - L - Q - V - L - A;
(ii) G - D - L - Q - V - L;
(iii) D - L - Q - V - L - A;
(iv) D - L - Q - V - L; and
(v) antigenically active modified sequences (as hereinafter defined) based on any one of the sequences (i) to (iv) above.

According to a second aspect of the present invention, there is provided a synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, type A_{10} (A_{61}), characterised in that at least a portion of said

peptide is selected from amino acid sequences of the group consisting of:

(i) G - D - L - G - S - I - A;
(ii) G - D - L - G - S - I;
(iii) D - L - G - S - I - A;
(iv) D - L - G - S - I; and
(v) antigenically active modified sequences (as hereinafter defined) based on any one of the sequences (i) to (iv) above.

According to a third aspect of the present invention, there is provided a synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, type C1, characterised in that at least a portion of said peptide is selected from amino acid sequences of the group consisting of:

(i) D - L - A - H - L - T - A;
(ii) D - L - A - H - L - T;
(iii) L - A - H - L - T - A;
(iv) L - A - H - L - T; and
(v) antigenically active modified sequences (as hereinafter defined) based on any one of the sequences (i) to (iv) above.

The term "antigenically active modified sequences" as used herein, is used to describe sequences of amino acids which are based on any one of sequences (i) to (iv) of the first, second or third aspects of the invention as described above, but in which one of the amino acids in the said sequence is

replaced by another amino acid to provide a modified sequence which is antigenically active.

Antigenically active modified sequences in accordance with the first aspect of this invention include hexapeptide sequences based on the formula:

G - D - L - Q - V - L
(1) (2) (3) (4) (5) (6)

in which:

G at position (1) is replaced by A, H, I, K, M, N, P, Q, S or T; or

D at position (2) is replaced by A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V or Y; or

Q at position (4) is replaced by D, K, A, M, E, N, S or R; or

V at position (5) is replaced by A, D, E, F, I, K, L, M, N, Q, R, S, T or Y.

Antigenically active modified sequences in accordance with the second aspect of this invention include hexapeptide sequences based on the formula:

G - D - L - G - S - I
(1) (2) (3) (4) (5) (6)

in which:

G at position (1) is replaced by A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; or

D at position (2) is replaced by L; or
G at position (4) is replaced by A; or
S at position (5) is replaced by T, G or A; or
I at position (6) is replaced by A, C, D, E, F, K, L, M, Q, R, S, T, V, W or Y.

Antigenically active modified sequences in accordance with the third aspect of this invention include hexapeptide sequences based on the formula:

D - L - A - H - L - T
(1) (2) (3) (4) (5) (6)

in which:

D at position (1) is replaced by H or V; or
H at position (4) is replaced by A, C, D, E, F, K, L, M, N, Q, R, S, T, V or Y; or
T at position (6) is replaced by S.

In arriving at this invention, the concurrent chemical synthesis of all possible hexapeptides representing the total sequences of the VP1 coat proteins of FMD virus, types O_1, A_{10} (or A_{61}) and C_1, with the overlap of five amino acids between peptides in juxtaposition in the sequence has been completed using the solid-phase synthesis technique described above. The synthetic peptides, still attached to the support used for their synthesis, have been tested for antibody-binding activity by ELISA to produce a complete map of all the reactive sequences of the VP1 proteins at a resolution of six amino

acids. A peptide length of six amino acids was chosen, taking into account the following factors:

1. antibody interactions with hexapeptides have been demonstrated;
2. the longer a peptide the greater the possibility of its folding with a secondary structure, this folding not necessarily being that occurring in the native protein;
3. during synthesis the yield of the desired peptide decreases as the number of coupling reactions increases; and
4. the smaller the sequence synthesized the greater the precision in specifying the bounds of a detected active sequence within the whole sequence.

As set out above, the peptides of the present invention are characterised in that at least a portion of the peptide contains an amino acid sequence selected from the groups (i) to (v) described above. Tests which have been carried out, particularly utilising the ELISA technique, have established the activity of these amino acid sequences by their reactivity with antisera. It should be noted, however, that whilst the synthetic peptides of this invention may comprise sequences which are only 5, 6 or 7 amino acids long, the present invention also extends to synthetic peptides in which one or more additional amino acids are included on either or both ends of the defined sequence, that is either on the carboxyl (-COOH) end or on the amino ($-NH_2$) end or

both. While such additional amino acids may not play any direct role in enhancing the immunogenic activity of the defined amino acid sequence, they may act, for example, as spacer amino acids to space the antigenically active amino acid sequence from the free carboxyl and/or free amino ends of the sequence.

Following identification of the antigenically active amino acid sequences of the VP1 proteins, the modified sequences in accordance with this invention have been similarly synthesised and tested utilising the ELISA technique. As previously described, each of these modified sequences was derived by replacing one amino acid in the original sequence by another amino acid.

The peptides of the present invention can be chemically synthesized from their constituent amino acids. This synthesis may be carried out, for example, by the Merrifield solid-phase method, as described in J.A.C.S. 85: 2149-2154 (1963). In the Merrifield solid-phase method, the C-terminal amino acid is attached to chloromethylated polystyrene-divinylbenzene copolymer beads. Each subsequent amino acid, with a suitable protecting group if necessary, is then added sequentially to the growing chain. As described in the Merrifield article, the protective group may be, for example, a t-butyloxycarbonyl or carbobenzoxy group. By the procedure of coupling, deprotection, and coupling of the next amino acid, the desired amino acid sequence

and chain length can be produced. As a final step, the protective group is removed from N-terminal amino acid, and the C-terminal amino acid is cleaved from the supporting beads, using a suitable reagent such as trifluoroacetic acid and hydrogen bromide. Alternatively, the synthesis can be carried out by the solid-phase method, in which the synthetic peptide structure is built up on a solid-phase carrier which comprises a polymeric material, for example a polyethylene rod or pin, having a vinyl monomer graft polymerised thereto, for example by γ-radiation of acrylic acid, acrylonitrile or acrylamide monomers. A mono- protected biamine such as lysine or lysine-alanine is then reacted with the polymeric chain formed on the solid-phase carrier and the synthetic peptide then built up by sequential reaction of amino acids in the same manner as in the Merrifield method described above.

It should be noted that whilst the final step in both the methods referred to above is the removal of the synthetic peptide from the solid-phase carrier or support by a cleaving reaction using a suitable reagent, for some uses of the peptides of the present invention it is convenient if not essential for the chemically synthesized peptides to be used in the form of a product in which the peptide is coupled or linked to a solid-phase carrier or support. For such uses, of course, the final cleaving step is omitted. Such antigenically active, chemically synthesized peptides which are immobilised on a

solid-phase carrier or support are of particular utility in the performance of immunochemical assays, such as enzyme immunoassays (EIA).

The immunogenic synthetic peptides of the present invention may provide the basis upon which the formation of synthetic vaccines against foot-and-mouth disease virus can be developed. Such synthetic vaccines would have particular merit insofar as they could be manufactured so as to be free of amino acid sequences corresponding to the entire amino acid sequences of the viral protein, and also free of biologically produced materials and of active or inactivated viral residues. The use of synthetic peptides as the basis for vaccines is discussed, for example, by Beale, J. in Nature 298 14-15 (1982), and by Sutcliffe, J.G. et al in Science, 219, 660-666 (1982). In formation of a synthetic vaccine, the synthetic peptides of the present invention could be used either alone, in combination, and/or in association with a physiologically acceptable carrier and/or adjuvant. The synthetic peptides of the present invention also have potential for use in a number of other applications in the immunological field, including use in diagnostic and other immunological testing procedures, particularly in immunochemical assays such as enzyme immunoassays.

Further details of the peptide compounds of this invention, their method of preparation and their

antigenic activity are illustrated by the following Examples:

EXAMPLE 1.

A. Preparation of hexapeptides.

The 213-amino acid sequence of VP1 (FMDV, type O_1) as translated by Kurz, C. et al., Nucleic Acid Research 9, 1919-1931 (1981) was subdivided into all possible hexapeptide units, and each hexapeptide unit was synthesized on a polyethylene support in the same orientation, and with a 2-long amino acid spacer as illustrated in Figure 1.

Polyethylene rods immersed in a 6% v/v aqueous solution of acrylic acid were γ-ray irradiated at a dose of 1 Mrad (see Muller-Schulte, D., Horster, F.A., Polymer Bulletin 7, 77-81 (1982)). Using conventional methods of solid-phase peptide chemistry (see Erickson, B.W., Merrifield, R.B. in "The Proteins", Vol.2, 255-257, Academic Press, New York (1976); Meienhofer, J., in "Hormonal Proteins and Peptides", Vol.2. 45-267, Academic Press, New York (1973)), N^{α}-t-Butyloxycarbonyl-L-Lysine methyl ester was coupled to the polyethylene polyacrylic acid (PPA) via the N -amino group of the side-chain. This was followed by the coupling of Boc-Alanine, to complete a peptide-like spacer. Amino-substitution of the support was determined by reacting NH_2-Lysine(OMe)-PPA

with C^{14} labelled butyric acid, and was found to be 8-10 nmoles/rod.

Successive amino acids were added by conventional solid phase peptide synthesis as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the t-butyloxycarbonyl (Boc) protecting group, the terminal amino group was acetylated with acetic anhydride in a dimethylformamide(DMF)/triethylamine mixture. All dicyclohexyl carbodiimide-mediated coupling reactions were carried out in DMF in the presence of N-hydroxy benzotriazole. The following side-chain protecting groups were used; O-benzyl for threonine, serine, aspartic acid, glutamic acid and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methyl benzyl for cysteine and 1-benzyloxycarbonylamido-2,2,2,-trifluoroethyl for histidine. Side-chain deprotection was achieved by treatment with borontris(trifluoracetate) in trifluoroacetic acid for 90 minutes at room temperature (see Pless, J., Bauer, W., Angewante Chemie 85, 142 (1973)). After hydrolysis in HCl/propionic acid, analysis of sequences included in the synthesis as controls confirmed that coupling at each stage had occurred. Before testing by ELISA, rod-coupled peptides were washed several times in phosphate buffered saline (PBS).

B. Testing of Hexapeptides.

Antigenic profiles for the hexapeptides prepared as described in A. above are shown in Figure 2 as a vertical line proportional to the ELISA extinction obtained, over the number giving the location within the VP1 sequence of the peptide N-terminal amino acid. Antisera used to produce the different profiles as shown, were as follows:

(a) and (b) two different anti-intact virus particle, type O_1;

(c) anti-intact virus particle, as used in (b), after absorption with purified complete virus, type O_1;

(d) anti-virus-subunit, type O_1;

(e) anti-VP1, type O_1 and

(f) anti-intact virus particle, type C_1.

The enzyme-linked immunosorbent assay was used to test each rod-coupled peptide (RCP) for reactivity with each of the defined antisera described above. RCPs were pre-coated with 10% horse serum, 10% ovalbumin and 1% Tween-80 in PBS, to block non-specific absorption of antibodies, for 1 hour at 37°C. Overnight incubation at 4°C in antiserum diluted 1/40 in the preincubation mixture, was followed by 3 washes in 0.05% Tween-80/PBS. Reaction for 1 hour at 37°C with the appropriate anti-rabbit IgG immunoglobulin coupled to horse radish peroxidase, diluted 1/50,000 in the preincubation mixture, was again followed by extensive washing in PBS/Tween to remove excess conjugate. The presence of antibody was detected by reaction for 45 min with a developing solution (40 mg orthophenylenediamine, 20 µl of

hydrogen peroxide in 100 ml of phosphate buffer, pH 5.0), and the colour produced read in a Titertek Multiscan at 420 nm. After tests, peptides were washed three times at 37°C in 8M urea containing 0.1% 2-mercaptoethanol and 0.1% sodium dodecyl sulphate, followed by several washes in PBS to remove all traces of bound antibody. The RCPs were then ready for further testing with different antisera.

Anti-intact virus particle sera were prepared by immunising rabbits with 50 µg of inactivated, purified virus in complete Freund's adjuvant. The animals were bled 3-4 weeks after the single vaccination. Anti-virus-subunit serum (rabbit) was prepared by immunizing 3 times, 3-4 weeks apart, with 10 µg of acid-disrupted purified virus, initially in complete Freund's and subsequently in incomplete Freund's adjuvant. The polypeptide VP1 was separated from the mixture of proteins obtained from urea disrupted, purified virus, by iso-electric focusing. (see Barteling, S.J., Wagenaar, F., Gielkens, A.L.J., J.Gen.Virol. 62, 357-361 (1982).) After elution from the gel with 8M urea and dialysis against PBS, antiserum was raised as described for 12S above. Antiserum for scan (c) was that used for scan (b), but after absorption with purified virus (1500 µg complete virus was incubated with 1 ml of serum for 72 hours at 4°C), and all virus bound antibodies removed by centrifugation.

C. Identification of the virus particle-associated antigenic peptide.

Of the four anti-intact virus particle sera tested, scans (a) and (b) show the extremes in the reactivity patterns found. Large quantitative differences in the response to an identical antigen preparation have been reported before, however, these scans highlight the variability possible in the antibody composition between sera. From an examination of scans (a), (b) and (c), antibody reactive with peptides numbers 146 and 147 are present in whole anti-intact virus sera, but absent after absorption with purified virus. These same antibodies are not observed in the anti-subunit sera, scan (d), and only weakly present in the anti-VP1 sera, scan (e). That some activity was found in the anti-VP1 sera, possibly accounts for the immunizing capacity, albeit weak, of the isolated protein. (see Kleid, D.G., et al., Science 214, 1125-1129 (1981).) It should be noted however that another anti-VP1 serum also tested, while retaining a strong activity in position number 148, showed nothing at positions numbers 146 and 147. The superimposition of scan (c) on scan (b) (absorbed compared to non-absorbed) shows that in addition to the loss of activity to peptides numbers 146 and 147, a reduction in activity to peptides numbers 5, 6 and 206 also occurred. Of these, activity to numbers 5 and 6 was not found in all the anti-intact virus sera tested, whilst number 206 activity was invariably present.

From these results, it is concluded that of the sequences found to be reactive, the pair at numbers 146 and 147, that is the hexapeptides Gly-Asp-Leu-Gln-Val-Leu (G - D - L - Q - V - L) and Asp-Leu-Gln-Val-Leu-Ala (D - L - Q - V - L - A), constitute the principal loci, with a lesser contribution from the locus at number 206, consistent with the observations of others. However, with respect to the loci at numbers 146-7, we do not distinguish between the two possibilities; one, that the active element is five amino acids long, i.e. the sequence common to both Asp-Leu-Gln-Val-Leu (D - L - Q - V - L); or two, that the active element is seven amino acids long, i.e. the combination of the two hexapeptides Gly-Asp-Leu-Gln-Val-Leu-Ala (G - D - L - Q - V - L - A).

EXAMPLE 2

A. Preparation of hexapeptides.

The 212 amino acid sequence of VP1 (FMDV type A_{10} or A_{61}) as given by Bachrach, H.L., et al, Office International des, Epizootics was subdivided into 207 hexapeptides. These hexapeptides were synthesised as described in Example 1 above with the exception that the side chain of arginine was protected by the p-methoxybenzene sulphonyl group.

B. Testing of hexapeptides.

Antigenic profiles for the hexapeptides are shown in Figure 3. The antisera used to produce the profiles were:-

(a) anti-intact virus particle type A_{10}

(b) anti-intact virus particle as used in (a) after adsorption with purified complete FMDV type A_{10}.

The testing of the hexapeptide and preparation of the sera were essentially as described in Example 1.

C. Identification of virus-particle associated active element.

By reasoning identical to that used in Example 1 it is concluded that the hexapeptides Gly-Asp-Leu-Gly-Ser-Ile (G - D - L - G - S - I) and Asp-Leu-Gly-Ser-Ile-Ala (D - L - G - S - I - A) are the principal loci for the antigenic determinant of the A-type of FMDV.

As in the case of FMD virus, type O_1, described in Example 1, we did not distinguish between these two sequences, and accordingly it is concluded that it is possible that the active sequence is five amino acids long, i.e. Asp-Leu-Gly-Ser-Ile (D - L - G - S - I), or that it is seven amino acids long, i.e. Gly-Asp-Leu-Gly-Ser-Ile-Ala (G - D - L - G - S - I - A).

EXAMPLE 3.

A. Preparation of hexapeptides.

The 210 amino acid sequence of VP1 (FMDV type C_1) as given by Robertson, H.L., et al., Journal of Virology, 46, 311-316 (1983) was subdivided into 205 hexapeptides. These hexapeptides were synthesised as described in Example 2 above.

B. Testing of hexapeptides.

Antigenic profiles for the hexapeptides are shown in Figure 4. The antisera used to produce the profiles were:-

(a) anti-intact virus particle type C_1

(b) anti-intact virus particle as used in (a) after absorption with purified complete FMDV type C_1.

The testing of the hexapeptide and preparation of the sera were essentially as described in Example 1.

C. Identification of virus-particle associated active element.

By reasoning identical to that used in Example 1 it is concluded that the hexapeptide Asp-Leu-Ala-His-Leu-Thr (D - L - A - H - L - T) is the principal locus for the antigenic determinant of the C-type of FMDV.

These results clearly show the potential of a systematic scanning of a polypeptide sequence. They point out the likely location of the active determinant encompassed within the peptide with which Bittle, J.L. et al, Nature 298, 30-33 (1982), obtained the successful protection in guinea pigs to a subsequent challenge by FMDV.

EXAMPLE 4

A. Preparation of Peptides containing Replacement Amino Acids.

The synthesis method of Example 1 was used to synthesize 120 hexapeptides, each consisting of five original amino acids of the antigenic hexapeptide G - D - L - Q - V - L (type 0_1), the other amino acid being systematically replaced with each of the 20 possible naturally occurring genetically coded amino acids. This replacement was performed at each of the six positions in the peptide in turn. Thus, the 120 peptides comprised six copies of the original sequence and 114 variations of the original sequence, each of the 114 variations differing in only one amino acid from the original sequence. This strategy is illustrated diagrammatically in Fig.5.

B. Testing of Peptides containing Replacement Amino Acids.

The ELISA test of Example 1 was used to test the 120 peptides against an antiserum known to react with the original sequence. The results are shown in Fig.6 and Table 1. In Fig.6, the antibody-binding activity for each peptide is shown as a vertical line proportional to the ELISA extinction obtained. Within a group of 20 lines the left-hand line corresponds to the substitution of the original residue by alanine (A), and the right-hand line is for the substitution by tyrosine (Y). Lines in between are in alphabetic order according to the single letter code for each amino acid. Every group of twenty lines corresponds to the complete replacement set for one of the six N-terminal residue positions in the hexapeptide G - D - L - Q - V - L. It can be seen that the amino acids at certain positions in the sequence can be readily replaced without loss of antigenicity, whereas other positions cannot accept replacement without partial or complete loss of antigenicity. Those peptides containing replacements which result in retained antigenicity are candidates for use in vaccine manufacture.

C. The preparation and testing methods of A. and B. above were repeated with the antigenic hexapeptides G - D - L - G - S - I (type A_{10}) and D - L - A - H - L - T (type C_1) - see Fig.5. The results are shown in Table 1.

EXAMPLE 5.

The selected antigenic peptides of each of the three FMDV types were synthesized with a variety of linking amino acids at the N-terminal end and with the linking amino acid lysine at the C-terminal end. The amino acids in the links do not occur in those positions in the native sequences. The synthesized peptides were coupled to a protein carrier and combined with an adjuvant for the purpose of animal immunisation. The carrier was keyhole limpet hemocyanin (KLH) and the adjuvant was either an oil adjuvant or aluminium hydroxide gel.

Table 2 gives the results of serological tests on immunised rabbits. It shows that in each case the rabbit produced a significant amount of antibody able to react with FMDV and able to neutralise the infectivity of FMDV.

EXAMPLE 6.

A protection test was carried out using guinea pigs as a model, since guinea pigs are susceptible to infection with FMDV. The peptide used for immunisation was C - G - D - L - Q - V - L - A - K, which is made up of the heptapeptide G - D - L - Q - V - L - A from FMDV type O_1 (residues 146-152), a cysteine linker at the N-terminal end and a lysine linker at the C-terminal end. To prepare the vaccine,

the peptide was coupled to KLH using maleimidobenzoyl N-hydroxysuccinimide ester, which links the peptide to the KLH via the cysteine side chain.

The KLH-peptide conjugate was then absorbed to an aluminium hydroxide gel and used to vaccinate the guinea pigs at a dose of 100µg peptide per animal. An unvaccinated group of guinea pigs served as controls. The animals were challenged 21 days after the single vaccination.

The results of challenge were as follows:

	Vaccinated	Unvaccinated
Fully protected	3	0
Partially protected*	2	0
Unprotected**	0	5
TOTAL	5	5

* 1 to 6 lesion score points on a scale of 0 to 12.

** Greater than 6 lesion score points on a scale of 0 to 12.

These results show that the short peptide sequence used can stimulate the immune system of a model susceptible animal to give a protective response against challenge by virulent FMDV.

EXAMPLE 7.

An example of the application of the present invention for diagnostic use is drawn from Fig.2. This shows that ELISA testing using support-coupled peptides (SCPs) is an extremely strain-specific tool for detecting FMDV antibodies. Whole antisera to FMDV type O_1 reacted with hexapeptides 146, 147 and 206 derived from the FMDV type O_1 sequence, whereas antiserum to type C_1 did not react at all. Control testing on the antiserum to FMDV type C_1 showed that, likewise, specific reaction only occurred with peptides derived from the C_1 sequence. Testing of the SCPs with other non-FMDV-specific control sera, and hyperimmune sera to other FMDV types, has also shown that no reaction occurs.

Animal sera can therefore be tested on selected FMDV SCPs and a positive reaction is diagnostic of a previous exposure of the animal to FMDV antigen.

Parent sequence	Position in sequence	Substituted amino acid																		
		A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
GDLGSI	1	66	66	49	61	63	105	107	60	87	50	67	69	15	65	57	63	60	27	101	59
Type A_{10}	2			118							28										
	3										88										
	4	29					98														
	5	12					27										106	97			
	6	47	96	28	81	87			85	108	108	97			108	162	97	72	55	23	19
GDLQVL	1	29					90	14	27	12		32	34	41	29		50				
Type O_1	2	22	21	143	95	110	28	65		12	65	15	58	10	69		38	62			
(48)	3										79										
	4			64	14								13		80						
	5	62		33	52				26		29	59			45		49	43	89		
	6										119										
GDLQVL	1	11					88	10		32		18	24	25	26		77	14			
Type O_1	2	37	12	136	92	137	52	62	21	87	81	37	89	49	80	29	63	104	60		21
(31)	3										88										
	4	60		117	52					68		53	49		102	10	45				
	5	52		40	63	42			56	82	68	88	34		106	33	91	98	81		14
	6										105										
DLAHLT	1			54					131										135		
Type C_1	2										133										
	3	108																			
	4	272	68	367	307	159		95		253	50	104	99		224	101	238	262	94		77
	5										93										
	6																30	117			

TABLE 1 Antibody-binding activities are shown for all peptides which gave an extinction significantly above the background. Values for each peptide are expressed as a percentage of the mean activity of the six parent sequences synthesised as a part of each replacement set. Underlined activities correspond to the values obtained for the parent sequence. No activity was detected when the antiserum used was prepared against the heterologous FMDV type.

TABLE 2

Summary of immunogenicity data for a peptide from each of the 3 sero-types of FMDV. Results given for rabit sera after 2 vaccinations of peptide to carrier [100µg dose]

Virus of Origin	Active Peptide	N-Terminal link	C-Terminal link	Anti-whole virus ELISA titre (log_{10})	Micro-neutralization titre (MNT) (log_{10})
FMDV Type O_1	GDLQVLA [146-152]	C-	-κ	2.9	1.0
		CS-	-κ	1.3	1.0
		CHS	-κ	2.3	1.0
FMDV Type A_{10}	GDLGSIA [144-150]	C-	-κ	1.4	1.0
		CS-	-κ	2.3	1.7
		CHS-	-κ	3.4	2.5
FMDV Type C_1	DLAHLTA [143-149]	C-	-κ	3.1	1.8
		CS-	-κ	2.0	1.3
		CHS	-κ	2.8	1.9

1. N-Terminal and C-Terminal additions were used to facilitate the coupling of the active peptide to the carrier protein Keyhole Limpet Haemacyanin (KLH)
2. ELISA titres were obtained using purified homologous virus as the test antigen.
3. MNT tests were carried out as quantal assays using microtitre trays. Dilutions of serum were incubated with 100 median infectious doses of homologous infectious virus before inoculation onto BHK cell monolayers.

It will, of course, be recognised that many variations and modifications may be made to the detailed description of the method of the present invention given above without departing from the method of the invention as broadly described herein.

CLAIMS:

1. A synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, characterised in that at least a portion of said peptide is selected from the group consisting of five-, six- or seven-long antigenically active amino acid sequences of said VP1 protein, and antigenically active modified sequences thereof.

2. A synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, Type O_1, characterised in that at least a portion of said peptide is selected from amino acid sequences of the group consisting of:

(i) G - D - L - Q - V - L - A;
(ii) G - D - L - Q - V - L;
(iii) D - L - Q - V - L - A;
(iv) D - L - Q - V - L; and
(v) antigenically active modified sequences (as herein defined) based on any one of the sequences (i) to (iv) above.

3. A synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, type A_{10} (A_{61}), characterised in that at least a portion of said peptide is selected from amino acid sequences of the group consisting of:

(i) G - D - L - G - S - I - A;

(ii) G - D - L - G - S - I;
(iii) D - L - G - S - I - A;
(iv) D - L - G - S - I; and
(v) antigenically active modified sequences (as herein defined) based on any one of the sequences (i) to (iv) above.

4. A synthetic peptide which displays the antigenicity of the VP1 protein of foot-and-mouth disease virus, type C1, characterised in that at least a portion of said peptide is selected from amino acid sequences of the group consisting of:

(i) D - L - A - H - L - T - A;
(ii) D - L - A - H - L - T;
(iii) L - A - H - L - T - A;
(iv) L - A - H - L - T; and
(v) antigenically active modified sequences (as herein defined) based on any one of the sequences (i) to (iv) above.

5. A synthetic peptide according to claim 2, characterised in that at least a portion thereof is selected from hexapeptide sequences based on the formula:

G - D - L - Q - V - L
(1) (2) (3) (4) (5) (6)

in which:
G at position (1) is replaced by A, H, I, K, M,

N, P, Q, S or T; or

D at position (2) is replaced by A, C, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V or Y; or

Q at position (4) is replaced by D, K, A, M, E, N, S or R; or

V at position (5) is replaced by A, D, E, F, I, K, L, M, N, Q, R, S, T or Y.

6. A synthetic peptide according to claim 3, characterised in that at least a portion thereof is selected from hexapeptide sequences based on the formula:

G - D - L - G - S - I
(1) (2) (3) (4) (5) (6)

in which:

G at position (1) is replaced by A, C, D, E, F, H, I, K, L, M, N, P, Q, R, S, T, V, W or Y; or

D at position (2) is replaced by L; or

G at position (4) is replaced by A; or

S at position (5) is replaced by T, G or A; or

I at position (6) is replaced by A, C, D, E, F, K, L, M, Q, R, S, T, V, W or Y.

7. A synthetic peptide according to claim 4, characterised in that at least a portion thereof is selected from hexapeptide sequences based on the formula:

D - L - A - H - L - T
(1) (2) (3) (4) (5) (6)

in which:

D at position (1) is replaced by H or V; or

H at position (4) is replaced by A, C, D, E, F, K, L, M, N, Q, R, S, T, V or Y; or

T at position (6) is replaced by S.

8. A vaccine for treating animals against infection by foot-and-mouth disease virus, comprising at least one synthetic peptide according to any one of claims 1 to 7.

9. A vaccine according to claim 8, further comprising a physiologically acceptable carrier and/or adjuvant.

10. A vaccine according to claim 8 or 9, wherein said at least one synthetic peptide is linked to said physiologically acceptable carrier.

11. A method of treating animals against infection by foot-and-mouth disease virus, which comprises administering to the said animals a vaccine according to any one of claims 8 to 10.

12. A diagnostic or other immunological testing system for detecting the presence of foot-and-mouth disease virus or of antibodies against foot-and-mouth disease virus in an animal, characterised in that it

comprises at least one synthetic peptide according to any one of claims 1 to 7 as an antigenically active component of said system.

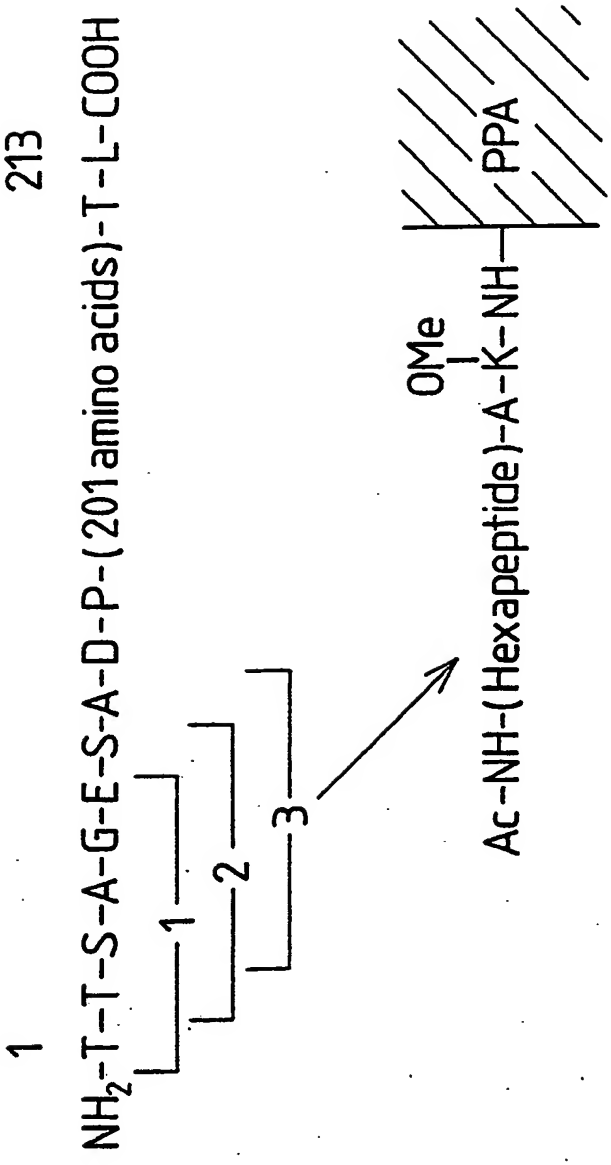


FIG 1

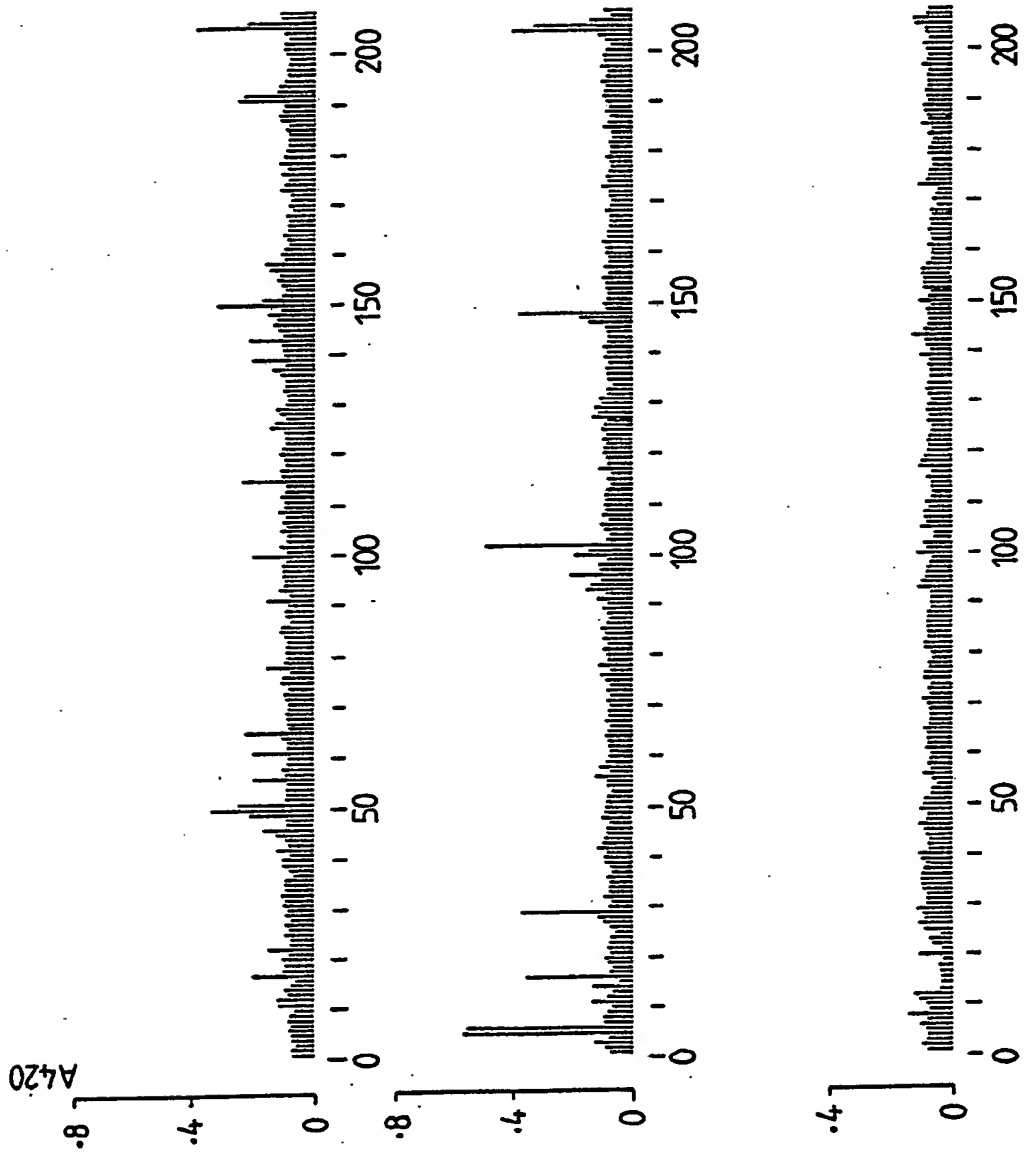


FIG 2d

FIG 2e

FIG 2f

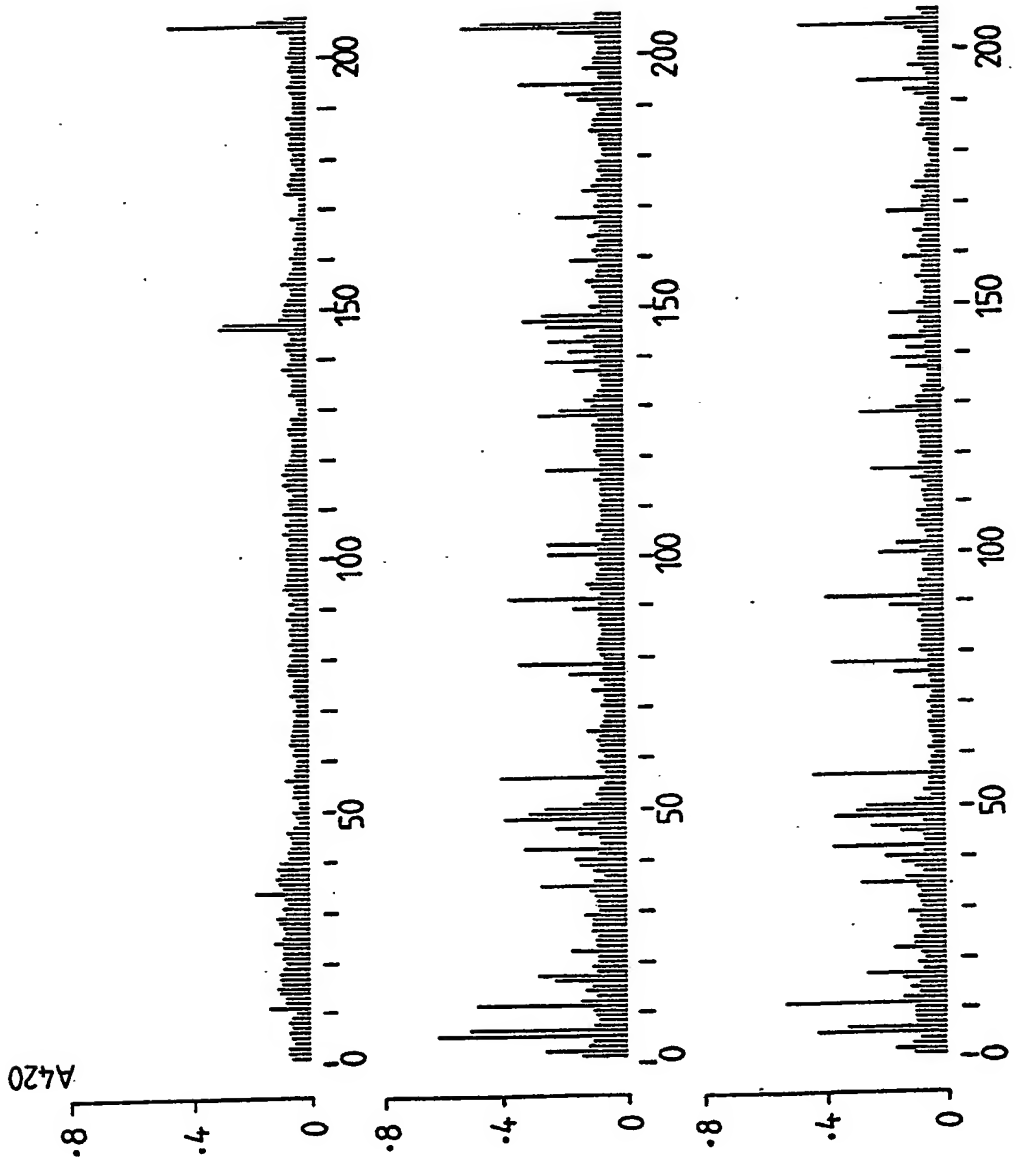


FIG 2a

FIG 2b

FIG 2c

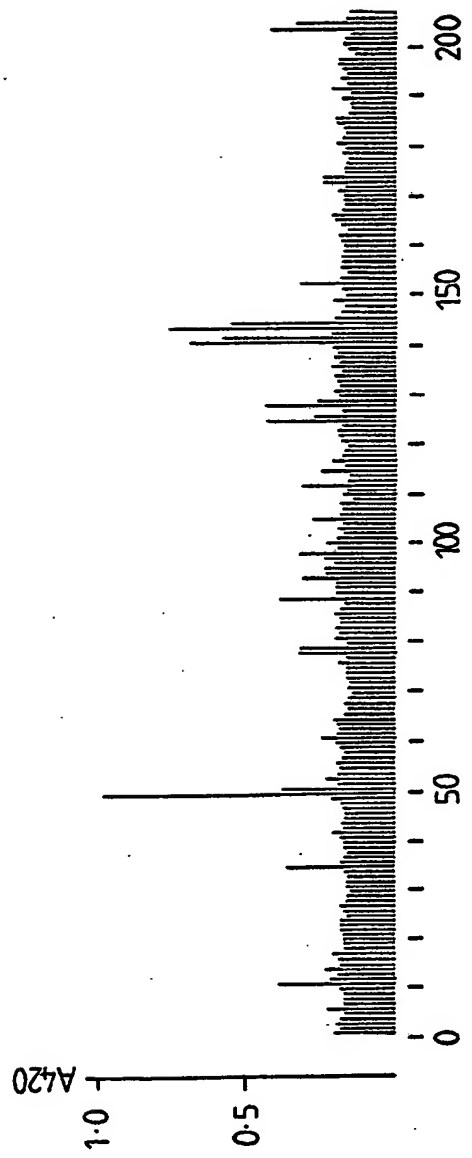


FIG 3a

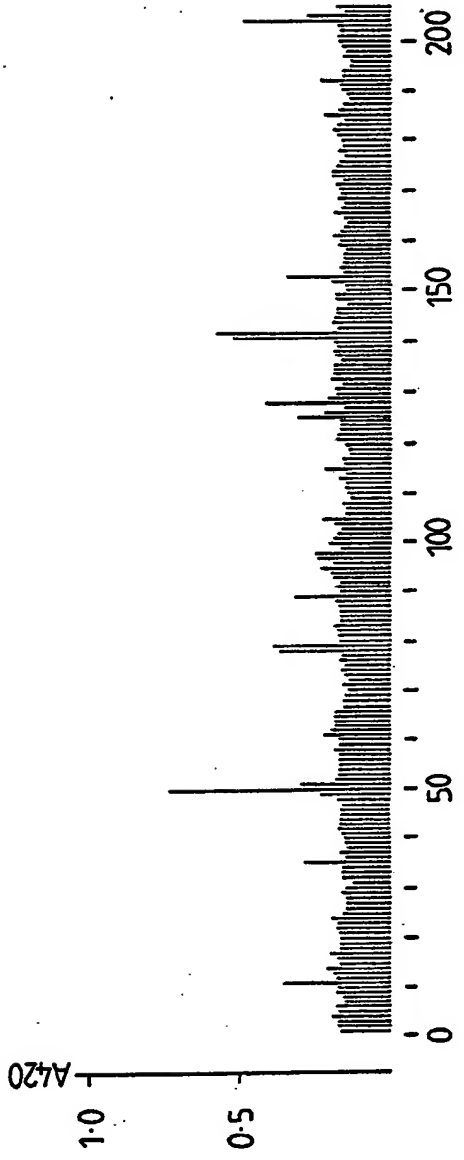


FIG 3b

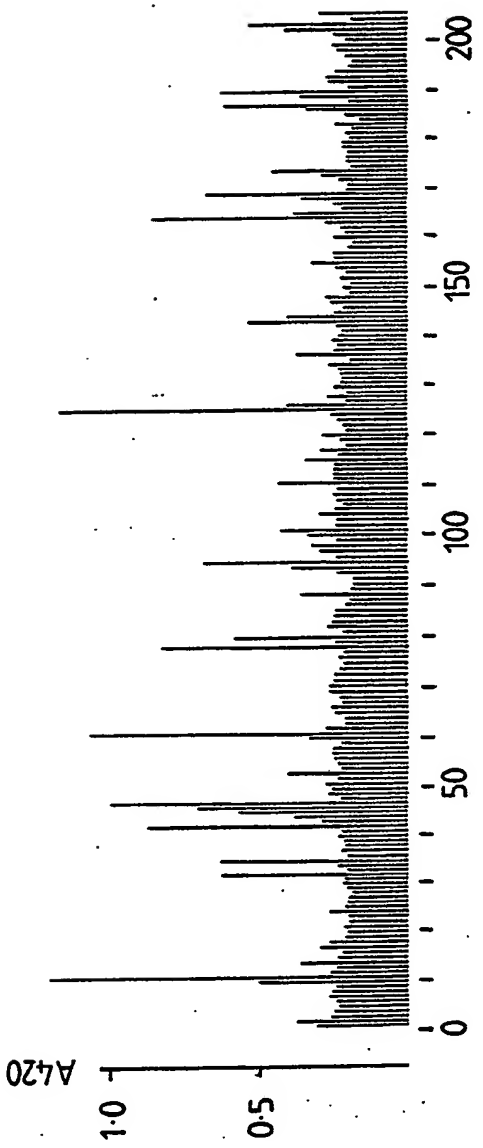


FIG 4a

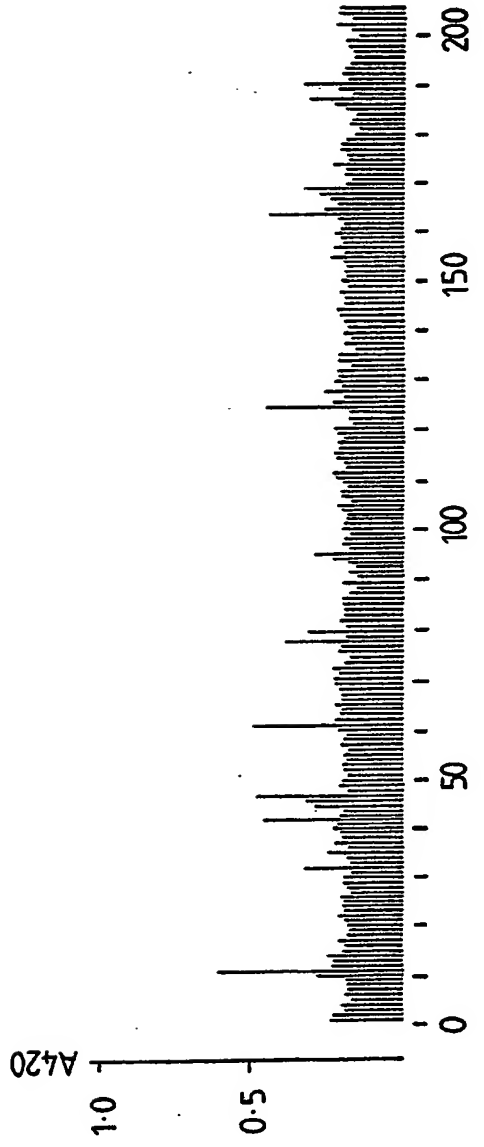


FIG 4b

FIG 5

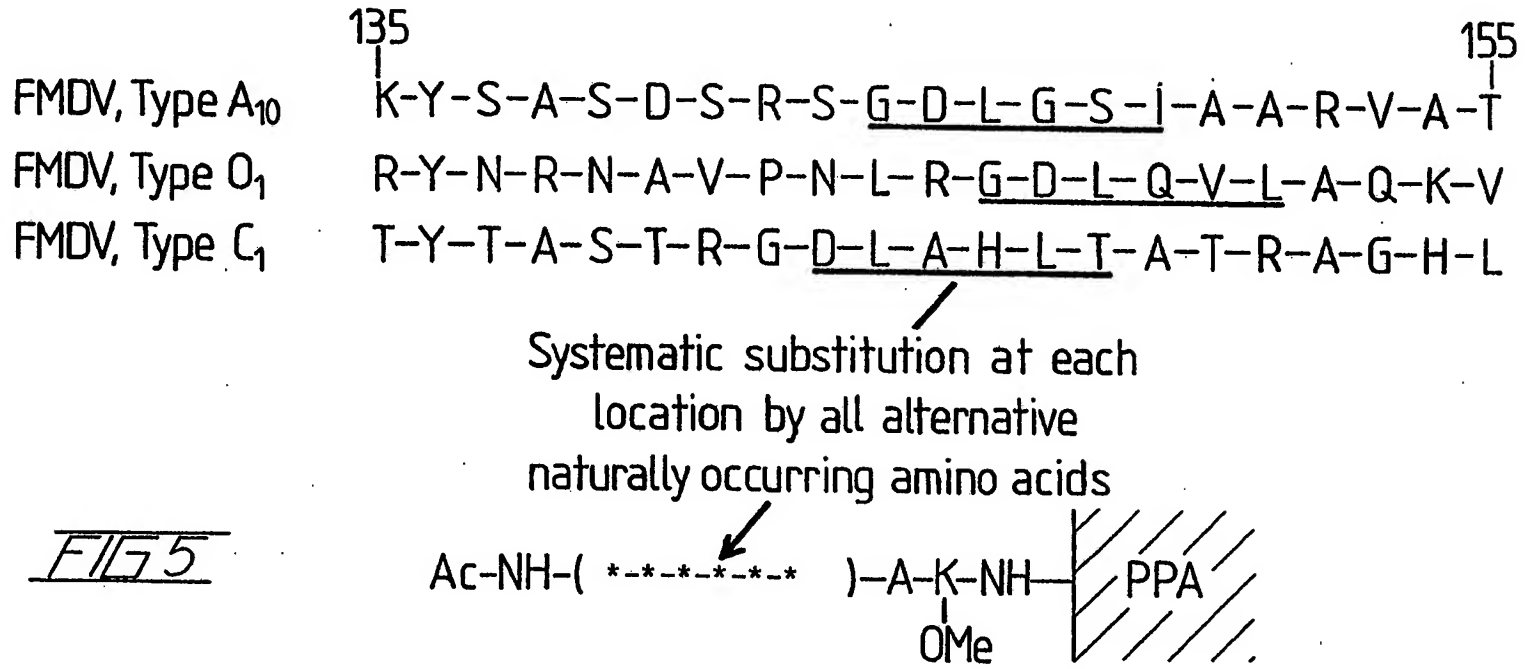
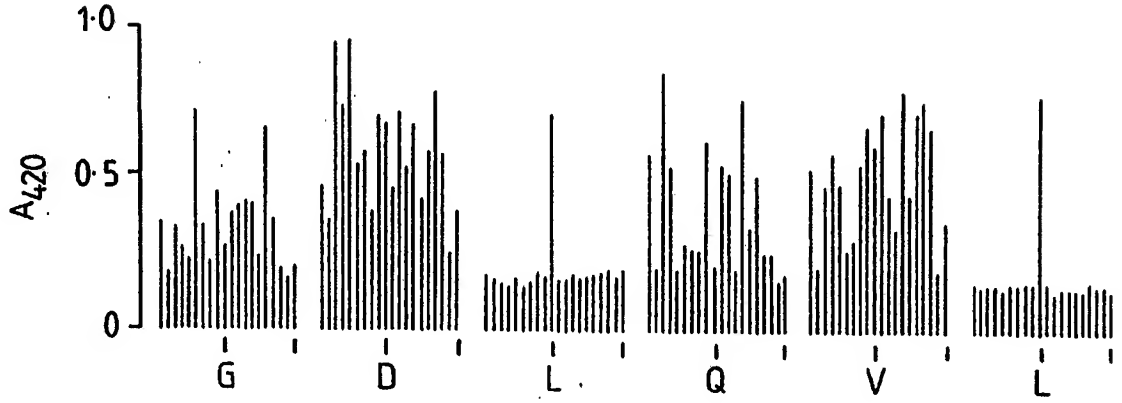


FIG 6



INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 84/00038

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] C07C 103/52, G01N 33/54, 33/56, C12Q 1/70, 1/28, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	C07C 103/52
US Cl.	260/112.5R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

AU:IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,Y	AU, A, 12553/83 (BIOGEN N.V.) 29 September 1983 (29.09.83)	(1-11)
P,Y	WO, A, 83/03547 (BITTLE et al.) 27 October 1983 (27.10.83)	(1-12)
Y	GB, A, 2 103 622 (GENENTECH INC.) 23 February 1983 (23.02.83)	(1-11)
Y	EP, A, 0048455 (THE WELLCOME FOUNDATION LIMITED) 31 March 1982 (31.03.82)	(1-11)
Y	EP, A, 0040922 (BIOGEN N.V.) 2 December 1981 (02.12.81)	(1-11)
A	Nature, Volume 290, issued 30 April 1981, BOOTHROYD et al., "Molecular Cloning of Foot and Mouth Disease Virus Genome and Nucleotide Sequences in the Structural Protein Genes", see pages 800 to 802	
A	Nucleic Acids Research, Volume 9, No. 8, issued 1981, KURZ et al, "Nucleotide Sequence and Corresponding Amino Acid Sequence of the Gene for Major Antigen of Foot and Mouth Disease Virus", see pages 1919 to 1931	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
17 May 1984 (17.05.84)	24-05-84 24 MAY 1984
International Searching Authority [1]	**Signature of Authorized Officer [20]**
Australian Patent Office	A.S. Moore A.S. Moore

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
A	J. Gen. Virol, Volume 46, issued 1980, ROBSON et al. "Biochemical Aspects of Variation in Foot-and-Mouth Disease Virus" see pages 179 to 193	
A	Annales de Recherches Veterinaires, Volume 8, No. 1, issued 1977, BERNARD et al, "Comparison of the Immunosenic Potencies of Purified Inactivated Foot and Mouth Disease (Type O) Virus Particles With Controlled Amounts of VPI Protein" see pages 79 to 94	
A,P	Journal of Virology, Volume 48, No. 2, issued November 1983, CHEUNG et al. "Comparison of the Major Antigenic Determinants of Different Serotypes of Foot and Mouth Disease Virus" see pages 451 to 459	
A,P	FEBS Letters, Volume 157, No. 2, issued July 1983, CLARKE et al. "Synthetic Peptides Mimic Subtype Specificity of Foot and Mouth Disease Virus", see pages 261 to 264	
A	Nature, Volume 298, issued 1 July 1982, BITTLE et al. "Protection Against Foot and Mouth Disease by Immunization with a Chemically Synthesized Peptide Predicted from the Viral Nucleotide Sequence", see pages 30 to 33	
A	Scientific American, Volume 248, No. 2, issued February 1983, LERNER, "Synthetic Vaccines", see pages 48 to 56.	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 84/00038

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members					
WO	8303547	DK	5743/83	EP	105346	FI	834590
		ZA	832644				
GB	2103622	BR	8203507	DK	2688/82	EP	68693
		ES	513159	IL	66042	JP	58049321
		PT	75050	ZA	824174	ZW	11982
EP	48455	AU	75418/81	BE	890393	DE	3137300
		DK	4136/81	ES	505572	FR	2490239
		GB	2084583	JP	58041899	NL	8104297
		SE	8105516	ZA	816469	ZW	23181
EP	40922	AU	70455/81	BR	8102922	DK	2076/81
		ES	502089	ES	511491	GB	2079288
		IL	62833	JP	57056496	NO	811609
		PL	231111	PT	73021	ZA	813110
		ZW	11281				

END OF ANNEX